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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 5/06, 5/10, A61K 9/48

A2

(11) International Publication Number: WO 97/39107

(43) International Publication Date: 23 October 1997 (23.10.97)

(21) International Application Number: PCT/CA97/00231

(22) International Filing Date: 11 April 1997 (11.04.97)

(30) Priority Data:

60/015,310	12 April 1996 (12.04.96)	US
60/019,737	14 June 1996 (14.06.96)	US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: METHODS FOR INCREASING THE MATURATION OF CELLS

(57) Abstract

The present invention provides a method of increasing the maturation rate or proliferation rate of a cell utilizing microencapsulation techniques. The invention also provides a method of treatment of a subject having diabetes utilizing cells produced by the culture method described herein.

METHODS FOR INCREASING THE MATURATION OF CELLS

FIELD OF THE INVENTION

The present invention relates generally to maturating cells, in particular, neonatal islet cells. These cells are useful for transplantation, for example, into a subject suffering from a diabetic condition. These transplanted cells supplement or replace the diabetic subject's deficient pancreatic islet cells, allowing the patient to produce insulin in response to glucose without insulin injections.

BACKGROUND OF THE INVENTION

Insulin-dependent diabetes mellitus (Type I) is caused by the progressive destruction of the insulin-producing pancreatic islet cells, which eventually leads to life-long dependence by the diabetic subject or patient on insulin therapy. A major focus of diabetes research has been to develop a better treatment to correct or alleviate the symptoms of diabetes, thereby preventing the disabling complications of the disease.

One approach is to transplant isolated insulin-producing pancreatic islet cells into the diabetic subject. Clinical trials using certain types of islet transplantation have corrected the abnormally-high blood glucose levels in Type I diabetics, rendering the transplant recipients partially or totally independent of exogenous insulin therapy. For the patients which are now independent of the insulin therapy, the transplantation has restored euglycemia. Several cases have been reported wherein human islet allotransplantation has corrected basal hyperglycemia, rendering the recipients insulin-independent for varying periods of time (1-6). This recent success is attributable to the development of reproducible methods for isolating and purifying human islets (7-9) and the production of an adequate cell mass to achieve insulin-independence. If islet transplantation is to become a widespread treatment for Type I diabetics, however, the supply of donor organs must be increased.

SUMMARY OF THE INVENTION

The present invention provides a method for increasing the rate of proliferation of a cell comprising encapsulating the cell within a stabilizing matrix and culturing the resulting cell *in vitro*. Also, the rate of maturation of an undifferentiated cell can be increased via the encapsulation of that cell. Preferably, the cell can be encapsulated within a microcapsule to form a microencapsulated cell. Typically, the microcapsule comprises alginate or agarose, which forms a thin layer of material around the encapsulated cell. In one aspect of the invention, more than one cell type is encapsulated within the microcapsule.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides an *in vitro* method for enriching a population of preferred cells from a tissue source including treating the tissue source to form a preparation and then culturing the preparation *in vitro* with a serum-free basal media supplemented with a serum substitute, an agent to stimulate DNA synthesis, and an agent to promote survival of the selected cell type, thereby resulting in an enriched population of desired cells. The selected cell type can include, but is not limited to, differentiated endocrine cells, such as neuroendocrine cells and adrenal cells; pancreatic endrocrine cells, endocrine precursor cells, such as stem cells and duct cells; hepatocytes, and the like. Preferably, the cells enriched are differentiated endocrine cells or endocrine precursor cells, with insulin-secreting neonatal islet cells most preferred.

The source of tissue can be any type so long as it contains the selected or desired cells for enrichment, and/or contains precursor cells capable of becoming the desired cells during the culture process. For example, tissue can include all or part of the liver, pancreas, thyroid gland, reproductive glands, myocardial tissue, renal tissue, blood, and the like, or a preselected group of cells from within these organs. Most preferred as a tissue source is the pancreas, whereby it is isolated from a pig or human. Further, the source of the pancreatic tissue can be pre-adolescent, fetal or neonatal, with neonatal as the most preferred source.

The enriched population of preferred cells is substantially enriched as described herein. The term "substantially enriched" as used herein means a population of selected cells wherein the majority of or at least about 90% of the cells are the selected cell type. For example, enriched aggregates of neonatal islet cells contain about 5% or less fibroblasts or pancreatic exocrine cells and most preferably contain about 2-5% or less of such cells.

In the present method, the tissue is typically prepared by partial or full digestion, as necessary, using an agent or mixture of agents, such as proteases, collagenases, other enzymes, such as disphase, and the like to form a digest. Collagenase, such as Type V,

as the cell survival promoting agent due to its cytoprotective effect of islet cells during culture and its ability to enhance islet cell replication.

The preferred supplements are present at about the following concentrations in the media: albumin from about 0.1% to about 1.0% weight/volume; IMBX from about 5 to about 100 µmol/liter; nicotinamide from about 0.5 to about 20 mmol/liter, and glucose from about 6 to about 30 mmol/liter. The digest is typically cultured at a temperature from about 20° to about 39°C for about 7 to about 20 days in humidified air. Most preferred, the digest is cultured for about 9 days at a temperature of about 37°C.

The serum-free tissue culture or basal media is a commonly used liquid tissue culture media that is free of serum. The media of the invention utilizes these media in combination with selected supplements or components to create a novel media to culture, enrich and allow the proliferation of, for example, the islet cells *in vitro*. Basal media useful in the culture method of the invention is any serum-free tissue culture media known in the art, including Media 199 (Gibco), CMRL 1066 (Gibco) media, Ham's F10 (Gibco) tissue culture media, and the like. These media also contain various ingredients, for example, amino acids, vitamins, inorganic salts, buffering agents, and energy sources. Purified molecules, such as hormones, growth factors, transport proteins, trace elements, antibiotics, and substratum-modifying ingredients optionally can be included in the media.

In a preferred embodiment, the invention comprises a serum-free basal media supplemented with albumin, IBMX, nicotinamide, and glucose. The pancreatic digest from one or more neonatal pig pancreas is preferably cultured with Ham's F10 tissue culture media, supplemented with about 10 m mol/liter of glucose, about 50 µmol/liter of IBMX, about 0.5% weight/volume of bovine serum albumin (BSA), and about 10 mmol/liter of nicotinamide. The media can optionally be supplemented with amino acids, such as L-glutamine, and antibiotics, such as penicillin and streptomycin. The

infect or transform the neuroblast (*Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

Various viral vectors which can be utilized for transfer of genes to the islet cells as taught herein include adenovirus, herpes virus, vaccinia, and preferably, an RNA virus such as a retrovirus. Retroviruses are useful particularly in the case of dividing cells. Therefore, the method of the invention, which provides a means for producing dividing and/or differentiated neonatal islet cells, provides cells that are susceptible to retrovirus infection. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). For human cells, preferably gibbon ape leukemia virus (GaLV) is utilized. A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated.

Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus (*gag*, *env*, and *pol* genes) under the control of regulatory sequences within the long terminal repeat (LTR). These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal include, but are not limited to Ψ2, PA317, PA12, CRIP and CRE, for example. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced. The vector virions produced by this method can then be used to infect a tissue cell line, such as NIH 3T3 cells, to produce large quantities of chimeric retroviral virions.

into a suitable recipient. Glucose tolerance testing, as described herein, can be used to monitor the effectiveness of the islet transplant.

Any of the transplantation or implantation procedures known in the art can be utilized. For example, the selected cells or cells of interest can be surgically implanted into the recipient or subject. Further, the cells can be administered in an encapsulated form or non-encapsulated form.

Transplantation or implantation is typically by simple injection through a hypodermic needle having a bore diameter sufficient to permit passage of a suspension of cells therethrough without damaging the cells or tissue coating. For implantation, the typically encapsulated or coated cells are formulated as pharmaceutical compositions together with a pharmaceutically-acceptable carrier. Such compositions contain a sufficient number of coated transplant cells which can be injected into, or administered through a laparoscope to, an animal, usually into the peritoneal cavity if islet cells are utilized. However, other transplantation sites can be selected depending upon the cells and desired biological effect; these sites include the liver, spleen, kidney capsule, omental pouch, and the like.

Typically, the number of transplanted islets is from about 5 to about 10 thousand per kilogram of body weight. For example, in mice approximately 1,000-3,000 islets are transplanted. The number of other cells, tissues, and the like will be calculated depending on their function.

While not required, it may be desirable to administer an immunosuppressive agent to a recipient of the islet cells, prior to, simultaneous with, and/or after transplantation. An agent such as Cyclosporine A (CsA) is preferable, however other immune suppressive agents can be used, such as rapamycin, desoxyspergualine, and like. These agents are administered to cause an immunosuppressive effect in the subject, such that the transplanted islet cells are not rejected by that subject's immune system. Typically, the

suspension of the material to be encapsulated, in a solution of monovalent cation alginate salt, *e.g.*, sodium. Droplets of the solution are then generated in air and collected in a solution of divalent cations, *e.g.*, $CaCl_2$. The divalent cations interact with the alginate at the phase transition between the droplet and the divalent cation solution resulting in the formation of a stable alginate gel matrix being formed. Generation of alginate droplets has previously been carried out by a number of methods. For example, droplets have been generated by extrusion of alginate through a tube by gravitational flow, into a solution of divalent cations. Similarly, electrostatic droplet generators which rely on the generation of an electrostatic differential between the alginate solution and the divalent cation solution have been described. The electrostatic differential results in the alginate solution being drawn through a tube, into the solution of divalent cations. For a general discussion of droplet generation in encapsulation processes, see, *e.g.*, M.F.A. Goosen, Fundamentals of Animal Cell Encapsulation and Immobilization, Ch. 6, pp. 114-142 (CRC Press, 1993).

Further, methods have been described wherein droplets are generated from a stream of the alginate solution using a laminar air flow extrusion device. Specifically, this device comprises a capillary tube within an outer sleeve. Air is driven through the outer sleeve and the polymer solution is flow-regulated through the inner tube. The air flow from the outer sleeve breaks up the fluid flowing from the capillary tube into small droplets. See U.S. Patent No. 5,286,495.

Microencapsulation also has been applied in the treatment of diseases by transplant therapy. While traditional medical treatments for functional deficiencies of secretory and other biological organs have focused on replacing identified normal products of the deficient organ with natural or synthetic pharmaceutical agents, transplant therapy focuses on replacement of that function with cell or organ transplants. For example, the treatment of insulin-dependent diabetes mellitus, where the pancreatic islets of Langerhans are nonfunctional, can be carried out by replacing the normal secretion of insulin by the islets in the pancreas. Insulin may be supplied either by daily administra-

able, biologically compatible layer of a polymer that binds chemically to the bonding bridge layer.

A method for introducing a second alginate gel coating to cells already coated with polylysine alginate is described in U.S. Patent 5,227,298. Both the first and second coating of this method require stabilization by polylysine.

Encapsulation methods applied to make these materials have comprised a procedure for forming droplets of the encapsulating medium and the biological material and a procedure for solidifying the encapsulating medium. Agarose encapsulated materials have been formed by chilling an emulsion of agarose droplets containing biological materials as shown by Nilsson, *et al.*, *Nature* 302:629-630 (1983) and Nilsson, *et al.*, *Eur. J. Appl. Microbiol. Biotechnol.* 17:319-326 (1983). Injection of droplets of polymer containing biological materials into a body of coolant such as concurrently liquid stream has been reported by Gin, *et al.*, *J. Microencapsulation* 4:329-242 (1987).

It is further envisioned that an undifferentiated cell that is microencapsulated or encapsulated with a stabilizing matrix, *i.e.*, block or hollow fibers, will mature at a rate faster than that of an unencapsulated cell. "Maturation" as used herein, means the ability of a cell to differentiate or to achieve a specific biological function or metabolic activity. Therefore, an encapsulated cell will mature at an increased rate over a cell not so encapsulated. Typically, the maturation rate will be at least about a 2-fold increase. Similarly, when an increase in proliferation rate is desirable, the rate will be at least about a 2-fold increase. Not being limited to a particular theory, it is believed that microencapsulation prevents cell aggregation thereby eliminating central necrosis of the aggregates, and allows the enclosed cells to grow and mature during culture. Another aspect of this invention is that encapsulation of a cell also increases the cell's rate of growth or proliferation in culture. For example, enclosing the neonatal islet cells within a microcapsule or stabilizing matrix allows them to mature, grow, and differentiate into insulin-secreting cells during the *in vitro* culture stage as well as after implantation.

Based upon existing methods of isolating fetal porcine islet tissue, a simple, reliable procedure was developed for the preparation of porcine neonatal islet cell aggregates with a reproducible and defined cellular composition. Following 9 days of *in vitro* culture, tissue from one neonatal pig pancreas yielded approximately 50,000 islet cell aggregates, consisting of primarily epithelial cells (57%) and pancreatic endocrine cells (35%). During the culture period, the total cell mass decreased initially, but subsequently increased 1.5-fold between days 3 and 9. Transplantation of grafts consisting of 3×10^5 cells (1000 aggregates) under the kidney capsule of alloxan-diabetic nude mice corrected hyperglycemia in 75% (10/13) of the animals, whereas, 100% (20/20) of recipients implanted with 6×10^5 cells (2000 aggregates) achieved euglycemia within 8 weeks post-transplantation. Nephrectomy of the graft bearing kidney at 14 weeks posttransplantation resulted in hyperglycemia in all recipients, and examination of the grafts revealed the presence of numerous well-granulated insulin- and glucagon-containing cells. The cellular insulin content of these grafts was 20 to 30-fold higher than at the time of transplantation. Further, aggregates cultured in alginate microcapsules and then transplanted into diabetic recipients corrected the diabetes within 1-7 days post-transplantation. These results indicate that the neonatal porcine pancreas can be used as a source of large numbers of viable islet cells, which have the potential for growth both *in vitro* and *in vivo*, and exhibit the metabolic capacity to correct diabetes.

ge-matched mice served as normal controls. All recipients entering this study exhibited blood glucose levels above 20 mmol/l. Blood samples were obtained from the tail vein for glucose assay (Medisense glucose meter, Medisense Canada, Mississauga, Ontario). Animals were maintained under Virus Antibody Free conditions in climatized rooms with free access to sterilized tap water and pelleted food.

Preparation and Culture of porcine NIC aggregates.

Each of the glands were cut into fragments of approximately 1-2 mm^3, then transferred to sterile tubes containing HBSS (supplemented as above) with 2.5 mg/ml collagenase, and gently agitated for 16-18 min. in a shaking water bath at 37°C. The digest was filtered through a nylon screen (500 um), washed four times in HBSS then placed into bacteriological petri dishes containing HAM's F10 tissue culture medium (10 mmol/l glucose, 50 umol/l IBMX, 0.5% BSA, 2 mmol/l L-glutamine, 10 mmol/l nicotinamide, 100 U/ml penicillin and 100 ug/ml streptomycin). Culture dishes were maintained at 37°C (5% CO2, 95% air) in humidified air for 9 days, with the medium and dishes changed the first day after isolation and the medium every second day thereafter.

For some experiments, following 8 days of culture, approximately 50% of the NIC aggregates from each preparation were microencapsulated with highly purified alginate (Metabolex, Inc.) producing uniform capsules of 250 to 350 µm in diameter. Encapsulated and non-encapsulated aggregates were then further cultured for 8 days (HAM's F10; 37°C) in the presence and absence of 5% (v/v) heat-activated autologous neonatal pig serum. Controls consisted of non-encapsulated aggregates cultured for 16 days in serum free HAM's F10.

Characterization of islet cell preparations.

Following the isolation procedure, and after 3 and 9 days of tissue culture, recovery and purity of the NIC aggregates was determined on the basis of cellular hormone, DNA, and amylase content. All measurements were assessed from duplicate aliquots of the islet cell suspensions. Hormone content was measured after extraction in 2 mmol/l acetic acid containing 0.25% BSA. Samples were sonicated in acetic acid, centrifuged (800 x g, 15

zymogen granules >500 nm in diameter), endocrine (presence of smaller granules typical for a, b, d, or pancreatic polypeptide cells), nongranulated (absence of secretory vesicles), or as damaged (ruptured plasma membranes and/or swollen organelles) (30,31). For immunohistochemistry, the avidin-biotin complex (ABC) method was used with peroxidase and diaminobenzadine as the chromagen. Sections (1 um) were affixed to glass slides by heat, the plastic resin removed with sodium methoxide and counter stained with Harris's hemotoxylin for 2 min., then subsequently stained separately for the presence of insulin- and glucagon-containing cells. In each experiment, a minimum of 15 aggregates randomly selected from 3 to 4 different sections were examined. Primary antibodies (Dako; Carpinteria, CA) included, guinea pig anti-porcine insulin (1:1000) and rabbit anti-glucagon (1:100); biotinylated secondary antibodies and the ABC-enzyme complexes were purchased from Vector Laboratories (Burlingame, CA). Primary antibodies were incubated for 30 min. (room temperature), while secondary antibodies were applied for 20 min.

For assessment of *in vitro* viability, the NICs secretory response to glucose was determined following 9 days of tissue culture by using a static incubation assay (27). The cultured fractions were recovered from the Petri dishes, washed and aliquots of 50 to 100 aggregates were incubated for 120 min. in 1.5 ml of RPMI medium supplemented with 2 mmol/l L-glutamine, 0.5% BSA and either 2.8 mmol/l glucose, 20 mmol/l glucose or 20 mmol/l glucose plus 10 mmol/l theophylline. Tissue and medium were then separated by centrifugation and assayed for their respective insulin contents. The insulin content of the medium was expressed as a percentage of the total content (*i.e.* tissue plus medium). Stimulation indices were calculated by dividing the amount of insulin release at 20 mmol/l glucose (+/- theophylline) by that released at 2.8 mmol/l glucose. In four independent experiments a portion of the freshly isolated NIC preparation was cultured for 9 days in the supplemented HAM's F10 medium, but without the addition of 10 mmol/l nicotinamide, in order to assess whether nicotinamide influenced the insulin secretory capacity of porcine neonatal β cells.

pelleted by centrifugation, and gently placed under the kidney capsule of Halothane-anesthetized nude mice with the aid of a micromanipulator syringe. Once the tubing was removed, the capsulotomy was cauterized with a disposable high-temperature cautery pen (Aaron Medical Industries, St. Petersburg, FL.). Transplanted mice were monitored for blood glucose levels once a week between 8:00 and 11:00 am. When the blood glucose level was ≤8.4 mmol/l, the graft was deemed a success.

Characterization of harvested NIC grafts.

At 14 weeks posttransplantation, NIC recipients underwent a nephrectomy of the graft-bearing kidney for morphological analysis or to determine insulin and glucagon contents of the harvested grafts. The grafts in four recipients receiving 2000 aggregates were however, not removed at this time, and these animals were monitored for an additional 7 months . Nephrectomized animals were subsequently monitored to confirm a return of hyperglycemia. The graft-bearing kidneys were immersed in Bouin's solution overnight and embedded in paraffin. Sections, 5 um thick, were then stained for the presence of insulin and glucagon containing cells, as described above. Pieces of native neonatal pig pancreas were also processed and analyzed according to this procedure. In two recipients, the graft and adjacent kidney tissue was fixed in glutaraldehyde then processed for electron microscopy. For hormone extraction, organs were homogenized and then sonicated at 4◦C in 10 ml of 2 mmol/l acetic acid (0.25% BSA). Following 2-h at 4°C, tissue homogenates were re-sonicated, centrifuged (8000 x g, 20 min.), then supernatants were collected and the pellets further extracted by sonication in an additional 8 ml of acetic acid. The second supernatant was collected after centrifugation, combined with the first supernatant, total volume was measured, and samples were assayed for insulin and glucagon content. The same procedure was also used to extract hormones from pancreases obtained from NIC recipients, normal control mice, and 1-3 day old neonatal pigs.

aggregate. Microscopically, NIC aggregates developed into spherical structures by the third day of culture and began to exhibit a translucent appearance similar to adult pancreatic islets. Tissue culture markedly reduced the percentage of exocrine cells, so that at 9 days post-isolation, <5% of the cells were identified as exocrine (Table II). These morphological findings are therefore consistent with the observed reduction in amylase content following culture. The nine day cultured preparations consisted of 35% structurally intact endocrine cells containing well-developed endoplasmic reticulum (Fig. 1). This percentage of endocrine cells was significantly higher than at the start of culture ($p<0.001$; Table II). In general, 9 day cultured aggregates contained numerous non-granulated epithelial cells (57%), as well as many duct-like structures were found in the aggregates (Fig. 1). A low degree of cellular damage was also observed in the electron micrographs (Table II). Immunohistological examination confirmed that 9 day cultured preparations consisted mainly of epithelial cells with the presence of 24% insulin-containing and 8% glucagon-containing cells scattered randomly throughout the aggregate (Fig. 2c and 2d). This random distribution of endocrine cells was also similar to that observed in the native neonatal porcine pancreas (Fig. 2a and 2b).

The quantity of aggregates recovered in each experiment was estimated using the method previously described for determining human islet equivalents (33). The mean yield obtained from one neonatal porcine pancreas following collagenase digestion and 9 days culture was 48,526±3125 aggregates (range = 28,210-90,966). Approximately 50% of the aggregates measured between 50 and 99 um, 40% were 100-149 um, and the remainder were either <50 um or ranged from 150-250 um.

EXAMPLE 3

INSULIN SECRETORY RESPONSIVENESS

The secretory activity of NIC aggregates cultured for 9 days in the presence or absence of 10 mmol/l nicotinamide was tested by comparing the percentages of cellular insulin that was released at low glucose (2.8 mmol/l), high glucose (20 mmol/l) and high glucose plus theophylline (10 mmol/l). No statistically significant differences were noticed in

glucagon content also confirmed that the mass of the 2000 aggregate grafts was correspondingly larger than the 1000 aggregate grafts.

EXAMPLE 5

TRANSPLANTATION OF NIC AGGREGATES INTO DIABETIC NUDE MICE

After alloxan administration, all NIC recipients exhibited blood glucose levels above 20 mmol/l. Diabetic controls (n=9) not receiving a graft were shown to survive for 11±4 days. All animals transplanted with 2000 NIC aggregates exhibited blood glucose values ±8.4 mmol/l within 8 weeks posttransplantation (Table V). This metabolic state was maintained over the 14 week follow-up period, and in 4 animals which were not sacrificed at 14 weeks, normoglycemia has been maintained for more than 11 months p-osttransplantation. On the other hand, 10 of the 13 animals implanted with 1000 aggregates achieved normoglycemia (blood glucose ±8.4 mmol/l) within the follow-up period (Table V). At 14 weeks posttransplantation, blood glucose values of those 1000 aggregate recipients not obtaining euglycemia were: 10.7, 12.2, and 14.4 mmol/l. Comparison with normal controls indicated that recipients of 2000 NIC aggregates exhibited significantly lower blood glucose levels at weeks 12 and 14 (Table V). These values did not, however, continue to decrease further, as those recipients (n=4) allowed to survive long term exhibited glucose levels of 5.2±0.6 at 11 months posttransplantation ($p<0.01$ vs. normal controls). No differences were measured between the mean glucose values of the 10 normoglycemic 1000-aggregate recipients and normal controls (Table V). In both transplanted groups, removal of the graft-bearing kidney for morphological examination or hormone extraction was followed by a rapid return to the diabetic state, indicating that the NIC grafts were responsible for the normoglycemic state.

Glucose tolerance tests were performed on normoglycemic mice 12 weeks post-transplantation, and when compared to normal control mice, recipients of 2-000-aggregates exhibited significantly lower glycemic values at all time points (Fig. 3). When the 1000-aggregate recipients were compared to normal controls, their blood

EXAMPLE 6

HORMONE CONTENT AND MORPHOLOGICAL CHARACTERIZATION OF NIC GRAFTS

Before implantation, NIC grafts contained on average 1.9 ug of insulin/1000 aggregates or 4.0 ug of insulin/2000 aggregates (Table IV). Fourteen weeks posttransplantation, considerably larger quantities of cellular insulin were recovered from the graft bearing kidneys (Table VI). There was however, no difference in the glucagon content between the harvested grafts and the grafts analyzed at the time of implantation (Table VI). The insulin content of grafts obtained from normoglycemic recipients implanted with 1000 aggregates contained 30-fold (63.7 ug) more insulin than what was initially transplanted (Table VI). Grafts retrieved from animals receiving 1000 aggregates and which did not achieve euglycemia, were shown to contain 12.2, 16.7, and 18.9 ug insulin. In animals transplanted with 2000 aggregates, grafts contained more than 20-fold (88 ug) more insulin than at the time of implantation (Table VI). The amount of insulin extracted from grafts obtained from recipients of 1000 and 2000 aggregates corresponds to, respectively, more than 74 and 141% of the pancreatic insulin content in aged-matched normal control mice (Table VI). Glucagon content, on the other hand, was similar to that found in pancreases of normal controls. The recipients pancreatic insulin content was less than 1% of that contained in normal control animals, whereas their glucagon content was similar to that in normal controls (Table VI).

Macroscopically, considerable growth of the NIC grafts was evident following 14 weeks posttransplantation. Immunohistological examination of the grafts revealed a highly vascularized tissue, consisting predominantly of well-granulated insulin- and glucagon-containing cells (Fig. 2 e,g and 2 f,h respectively). Epithelial cells were not frequently seen in the grafts. The β cells, which composed the major volume of the graft, were arranged in ductal-/tubular-like structures and the endocrine non-β cells were scattered randomly amongst the β cells. No marked differences in morphology were observed between the two transplant groups. In electron micrographs, donor endocrine

EXAMPLE 8

CO-ENCAPSULATION OF ISLETS WITH SERTOLI CELLS

In order to create an ectopic site, two thousand allogenic rat islets (Lewis) were co-encapsulated with allogenic Sertoli cells (Lewis) and implanted intraperitoneally into streptozotocin-induced diabetic (glycemia >20 mM) Wistar-Furth recipients (Table IX). All animals receiving encapsulated islet grafts without Sertoli cells achieved euglycemia (glycemia <8.4mM) within 2 days, but all returned to a diabetic state by 8-14 days posttransplantation (mean survival time 10.4±0.6 days). When islets were co-encapsulated with Sertoli cells, all animals again normalized within 2 days, and remained euglycemia for a period ranging from 74-98 days posttransplantation (mean survival time 85.0±4.9 days). These results demonstrate, that creation of a clinically applicable ectopic site that co-localizes islets and Sertoli cells, leads to a long-term protection of an islet allograft without the need for systemic immunosuppression.

Table II. Cellular composition of porcine neonatal islet cell aggregates during tissue culture.

		Cell composition (% of total)				Cell type (% positive)	
Culture Period	n	Endocrine	Nongranulated	Exocrine	Damaged	Insulin	Glucagon
Freshly isolated	7	7±2	11±1	74±2	8±1	5±1	2±1
3 Days	5	11±3	45±3‡	41±3‡	4±1*	8±1	3±1
9 Days	7	35±5†§	57±5‡¶	3±1‡¶	5±1	24±3‖"	8±1‡"

Values are means ± SE of n independent determinations. In each experiment, porcine NIC aggregates were prepared from 3 pancreases.

Cell composition was determined in electron micrographs and the percent glucagon and insulin containing cells was determined by immunohistochemistry, as described in Methods. Statistical significance of differences was calculated by one-way analysis of variance.

*p<0.05, †p<0.001, ‡p<0.0001 vs. freshly isolated.

§p<0.01, "p<0.001, ¶‖p<0.0001 vs. 3 Days.

Table IV. Composition of porcine neonatal islet cell grafts prior to transportation.

Graft	Content (μg/graft)			Purity (μg/μg)	
	Insulin	Glucagon	DNA	Insulin/DNA	Glucagon/DNA
1000 NIC aggregates	1.9±0.3	0.62±0.05	8.9±0.7	0.213±0.022	0.073±0.008
2000 NIC aggregates	4.0±0.4	1.32±0.11	18.9±1.5	0.219±0.025	0.072±0.007

Values are means ± SE of 10 independent experiments. In each experiment, porcine NIC aggregates were prepared from 3 pancreases.

Table VI. Hormone content after transplantation of porcine NIC aggregates.

Experimental Group	n	Hormone content (μg/organ) Insulin	Glucagon
Normal Controls			
pancreas	9	36.5±1.3	0.53±0.02
Recipients of 1000 NIC aggregates			
pancreas	8	<0.7	0.51±0.03
kidney	8	63.7±9.2*	0.40±0.07
Recipients of 2000 NIC aggregates			
pancreas	14	<0.7	0.46±0.06
kidney	14	88.0±5.6†‡	0.47±0.05

Values are means ± SE for n animals. All recipients exhibited normal glycemia and were analyzed at 14 weeks posttransplantation. Statistical significance of differences was calculated by one-way analysis of variance.
*p <0.05, †p <0.0001 vs. normal control pancreases.
‡p<0.05 vs. kidney grafts in recipients of 1000 NIC aggregates.

[b]One recipient died while normoglycemic.

[c]Aggregates were cultured for 16 days in serum free HAM's F10. When non-encapsulated NIC aggregates were initially cultured for 8 days without serum then subsequently cultured for an additional 8 days in HAM's F10 containing 5% neonatal pig serum, the aggregates clumped together becoming necrotic and were thus not of sufficient quality for transplantation.

Table VIII. Metabolic follow-up of diabetic nude mice transplanted with 2000 neonatal porcine islets.

		Weeks Posttransplantation							
Experimental Groups	Site	1	2	3	4	5	6	8	42
		Normoglycemic Animals[a]/Total number of recipients							
No Serum									
non-encapsulated	k.c.	0/8	0/8	2/8	4/8	6/8	7/8	8/8	8/8
encapsulated	i.p.	0/10	0/10	3/10	5/10	8/10	10/10	10/10	10/10
Plus Serum									
encapsulated	i.p.[b]	5/12	12/12	11/12	12/12	12/12	12/12	12/12	12/12
dissolved capsules	k.c.	2/9	9/9	9/9	9/9	9/9	9/9	9/9	9/9

k.c., kidney capsule; i.p., intraperitoneally.

[a]Normoglycemia defined as blood glucose values ≤ 8.4 mMl.

Table IX. Survival of Lewis rat islet allografts coencapsulated with Lewis Sertoli cells transplanted IP into diabetic Wistar-Furth recipients.

Alginate	Sertoli Cells (10^3)/Islet	n	Time Until Diabetes Recurrence (days) Individual
Mean±SEM			
MXG	None	15	8x3, 9x2, 10, 11x4,
10.4±0.6			12x2, 13, 14x2
MXG	5.5±0.2	5	74, 76, 82, 95, 98
85±4.9			

and the percentage of insulin-positive cells, the following equation can be used to calculate total β-cell mass in the isolated NIC aggregates:

$$\frac{\text{Total DNA content}}{\text{7.1 pg DNA/NIC}} \times \frac{\text{\% insulin positive cells}}{100} = \text{number of β-cells recovered per pancreas}$$

The number of β cells recovered from freshly isolated, 3-day, and 9-day cultured preparations is calculated to be 29.2, 8.5, 13.3 million cells per pancreas, respectively. Similarly, when considering the percentage of α-cells the calculated number of alpha cells is 11.7, 3.2, and 4.4 million cells per pancreas, respectively. The decrease in endocrine cell mass between the isolation and 3 days culture is likely the result of deleterious effects of the collagenase digestion and the presence of potentially cytotoxic proteases released from degenerating exocrine cells during culture. In contrast, the increase in both α and β cell mass (*i.e.* 38 and 56%, respectively) between day 3 and day 9 of culture, can possibly be explained by the growth or differentiation of new endocrine cells. Cellular insulin content also decreased significantly during the first 3 days of culture, and this is presumably related to the fall in β cell mass. However, between days 3 and 9 of culture, cellular insulin content decreased by 10%, yet β cell mass was shown to increase. Thus, even though new β cells were forming and contributing to the total insulin pool, their insulin stores as well as that of pre-existing β cells likely decreased as a result of secretory activity that exceeds the rate of insulin biosynthesis during culture. The recovery of cellular DNA decreased throughout the 9-day culture period, which is likely the result of the marked elimination of contaminating exocrine cells.

Many studies have indicated that the fetal β cell has a poor insulin response to glucose, which is rapidly converted to a more adult pattern after birth (22-25). In the present study, porcine neonatal β cells were capable of secreting significant amounts of insulin in response to a glucose challenge. This secretory capacity was further augmented when the NIC aggregates were pre-cultured in the presence of nicotinamide. These results are comparable to those described for islet cells prepared from the neonatal pig pancreas (1-3

Although the NIC grafts grown in the absence of culture in microcapsules were unable to correct diabetes immediately after transplantation, they eventually developed the capacity to establish and maintain euglycemia, likely because the relatively few β-cells implanted initially were subsequently supplemented by the growth and/or differentiation of additional new β cells. Interestingly, all recipients survived this hyperglycemic period, yet diabetic controls survived for only 11±4 days after alloxan treatment, suggesting that even in the first two weeks posttransplantation, NIC grafts produced sufficient insulin to keep recipients alive, although not euglycemia. We hypothesize that a hyperglycemic environment may be essential to inducing the growth and differentiation of new β-cells in our experimental model, and perhaps also in the clinical setting. At the time of implantation, the insulin content of NIC grafts corresponded to only 5 - 10% of the pancreatic insulin content found in aged matched normal controls. Following transplantation, the grafts insulin mass increased by >20-fold. Whether this increase in insulin mass was related to the birth of new β cells through differentiation of epithelial cells in the NIC aggregates and/or replication of existing β cells was not assessed in this study. It has however, been suggested that the major source of newly formed β cells in transplanted porcine islet cell clusters is from undifferentiated epithelial cells rather than from pre--existing β cells (11). Our morphological data indirectly support this concept, since at the time of implantation NIC grafts were composed predominately of epithelial cells, whereas several weeks after transplantation, few epithelial cells were detected and insulin--producing β cells now comprised the major volume of the graft. It cannot be excluded, however, that β cell proliferation did not contribute to at least some of the increased insulin content of the grafts, as electron micrographs indicated mitotic activity within some of the engrafted β cells. The use of semi-quantitative morphometric techniques, such as bromodeoxyuridine labeling and simultaneous immunostaining for islet hormones (15), should provide further insight into the growth kinetics of porcine NIC grafts. It is worthy to note that the grafts glucagon content did not increase following transplantation, suggesting that no additional growth of a-cells occurred. Similarly, in studies where fetal porcine islet cells were implanted into alloxan-diabetic nude mice, the frequency of glucagon containing cells markedly decreased after transplantation (11). These observations indicate that in immature islet cell grafts, continued growth and differentiation of endocrine non-β cells is limited, if not inhibited, when transplanted under the kidney capsule of alloxan-diabetic nude mice.

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WHAT IS CLAIMED IS:

1. A method for transplantation of endocrine cells into a subject, wherein the subject would benefit from the transplantation, comprising:

a) digesting a tissue source of differentiated endocrine or endocrine precursor cells to form a digest;

b) culturing the digest in vivo in a serum-free basal media supplemented with a serum substitute, an agent to stimulate DNA synthesis, and an agent to promote endrocrine cell survival, resulting in an enriched population of endocrine cells; and

c) transplanting the cells into the subject.

2. A method for transplantation of hepatocytes into a subject, wherein the subject would benefit from the transplantation, comprising:

a) digesting a tissue source of liver cells to form a digest;

b) culturing the digest in vivo in a serum-free basal media supplemented with a serum substitute, an agent to stimulate DNA synthesis, and an agent to promote hepatocyte cell survival, resulting in an enriched population of hepatocytes; and

c) transplanting the hepatocytes into the subject.

3. A method for transplantation of insulin-secreting neonatal islet cells into a diabetic subject comprising:

a) digesting a pancreas with collagenase to form a digest;

b) culturing the digest in vitro in a serum-free basal media supplemented with a serum substitute, an agent to stimulate DNA synthesis, and an agent to promote endocrine cell survival, resulting in an enriched population of insulin-secreting neonatal islet cells; and

c) transplanting the cells into the subject.

4. The method of Claim 3 wherein the islet cells are transfected with an exogenous foreign gene prior to transplantation.

ISOOCID: <WO___9739107A2_I_>

16. The method of Claim 14 wherein the cell is a porcine or human neonatal islet cell.

17. The method of Claim 14 wherein the microencapsulated cell is cultured in a serum-containing or serum-free media.

18. The method of Claim 14 further comprising co-encapsulating at least two cell types.

19. The method of Claim 18 wherein the cells are Sertoli cells and neonatal islet cells.

FIG. 2B

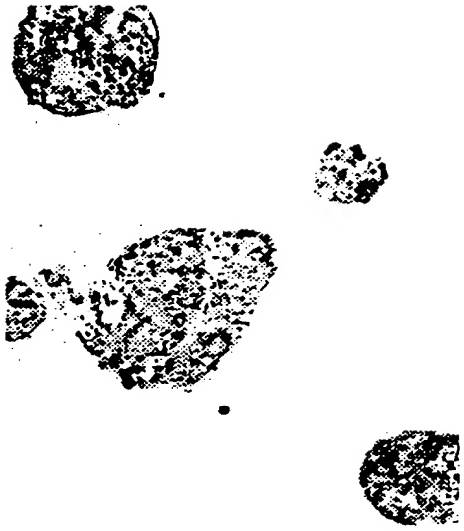

FIG. 2D

FIG. 2A

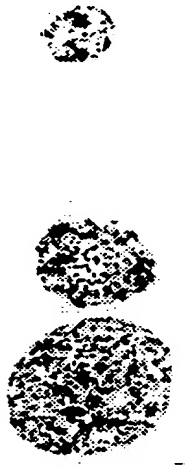

FIG. 2C

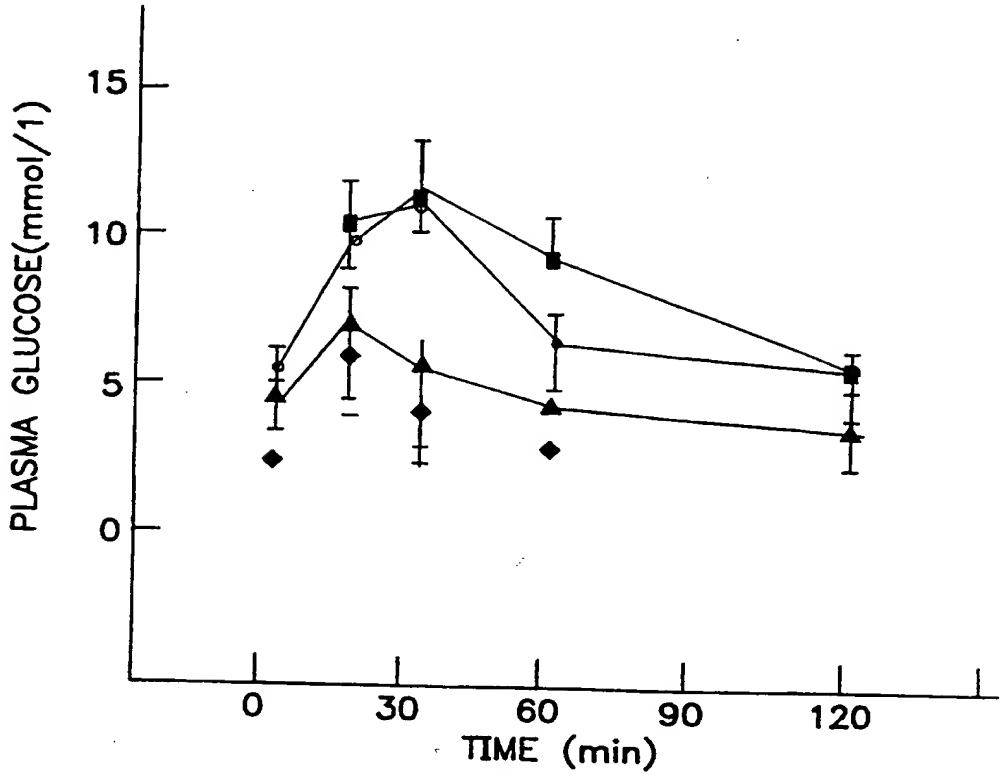

FIG. 3A

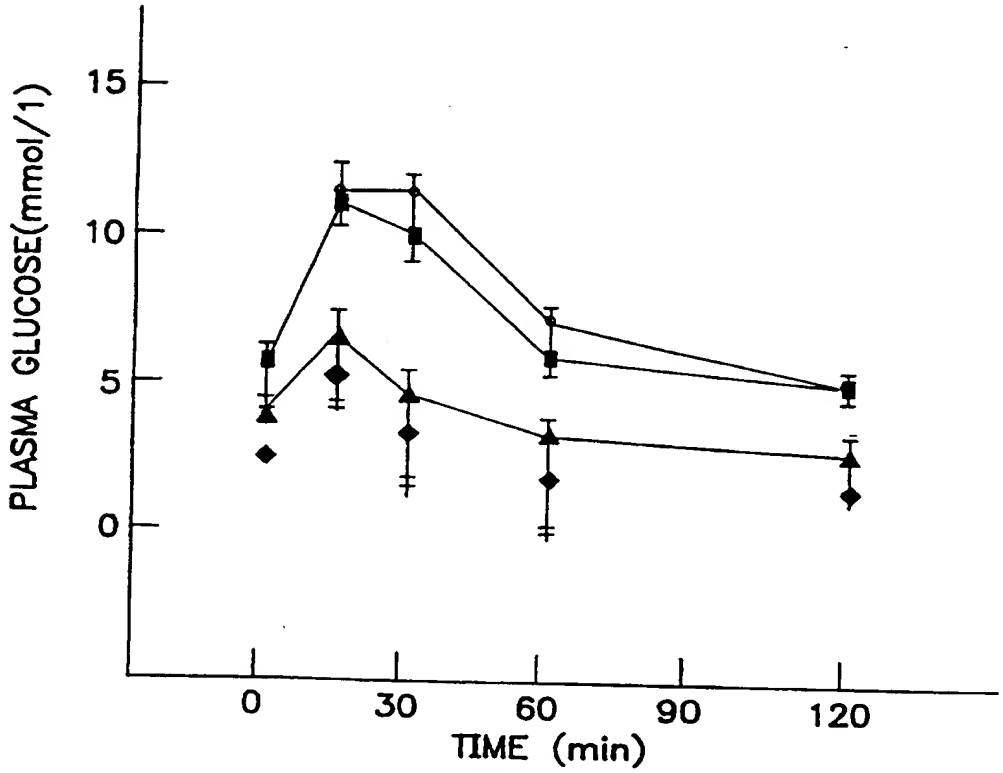

FIG. 3B

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/06, 5/08

A3

(11) International Publication Number: **WO 97/39107**

(43) International Publication Date: 23 October 1997 (23.10.97)

(21) International Application Number: PCT/CA97/00231

(22) International Filing Date: 11 April 1997 (11.04.97)

(30) Priority Data:

60/015,310	12 April 1996 (12.04.96)	US
60/019,737	14 June 1996 (14.06.96)	US

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report: 11 December 1997 (11.12.97)

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(54) Title: METHODS FOR INCREASING THE MATURATION OF CELLS

(57) Abstract

The present invention provides a method of increasing the maturation rate or proliferation rate of a cell utilizing microencapsulation techniques. The invention also provides a method of treatment of a subject having diabetes utilizing cells produced by the culture method described herein.

INTERNATIONAL SEARCH REPORT

Intern .sal Application No
PCT/CA 97/00231

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/06 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 363 125 A (HANA BIOLOGICS INC.) 11 April 1990 see abstract see page 4, line 20 - page 14, line 21 ---	1,3,5
X Y	EP 0 127 989 A (CONNAUGHT LABORATORIES LTD) 12 December 1984 see abstract see the whole document & US 4 673 566 A (CONNAUGHT LAB.) 16 June 1987 cited in the application --- -/--	1-3,7-9, 11,14, 15,17 12,13, 18,19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
10 September 1997	28.10.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/CA 97/ 00231

Box I Observations where certain claims were found unsear hable (Continuati n of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2 ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3 ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1) Claims 1-7: method for the preparation of a tissue prior to a transplantation, namely its selection, digestion, culturing cells, their preparation and transplantation.
2) Claims 8-19: use of encapsulation to obtain some particular effects like increased maturation and proliferation rates.

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.